Regulation of the Immune Response to Tumor Antigen

V. *Modulation of Suppressor T-Cell Activity* In Vivo

Mark I. Greene, MD, PhD, Linda L. Perry, MS, and Baruj Benacerraf, MD

Reduction of syngeneic tumor growth in primary tumor-bearing murine hosts has been accomplished using a variety of treatments designed to decrease endogenous suppressor cell activity or augment host effector responses. Selective interference with suppressor cell function can be achieved by *in vivo* administration of anti-thymocyte serums at critical times during the early stages of tumor development or by continuous treatment with antiserums directed to interact with I-J determinants on suppressor cells or suppressor factors. This later mode of therapy also results in a delay in tumor appearance when suboptimal doses of tumor are given. Preferential diminution of suppressor cell precursor activity has also been observed by pretreatment of tumor recipients with low doses of cyclophosphamide. Normal animals so treated are capable of adoptively transferring primarily helper-type activity to tumor-bearing recipients. Decreased tumor growth and prolonged host survival have also been achieved using BCG as a means of augmenting host effector potential. Thus, it is possible to inhibit tumor development in a murine model by modes of immunotherapy which may be relevant to the early treatment of certain human neoplasms. (Am J Pathol 95:159–170, 1979)

INVESTIGATIONS into the nature of host immune responsiveness to tumor antigens have revealed the existence of a population of suppressor T cells (STC) which are intimately involved in the regulation of this response. STC arise within 24 hours of antigen exposure [1] and can be found predominantly within the thymus and spleen of tumor-bearing animals.[1,2] These cells appear to act by limiting host cytolytic potential, although the point of interaction between suppressor cells and cells involved in the effector pathway has not been defined. Thus, STC and/or their antigen-specific suppressor factors [3,4] may interfere with the development of helper or amplifier cells or with the effector T cell itself.[1,3]

Certain investigations into the regulation of tumor development in animal models have focused on the elimination of suppressor cell activity, with the aim of allowing greater expression of host antitumor immunity. Already reported has been the reduction of suppressive influence through

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts.

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Address reprint requests to Mark I. Greene, MD, Department of Pathology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115.

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the administration of alloantiserums directed against determinants encoded by the I-J subregion of the murine H-2 complex.[5] The success of this mode of therapy in inhibiting tumor growth in primary tumor-bearing animals is believed to be due to a diminution of endogenous suppressor cell activity as a result of interaction between I-J antibodies and I-J determinant–bearing suppressor T cells or their factor(s).[5] Animals so treated are no longer capable of adoptively transferring suppression to immune recipients [5] and exhibit histologic evidence of enhanced tumor cell destruction *in vivo*.[6]

In this report we extend our earlier observations on the effect of anti I-J treatment on tumor development and we present data on additional modes of immunotherapy being examined as a means of reducing suppressor cell activity and/or enhancing host cytolytic capabilities. These include the administration of anti-thymocyte serums at critical times following tumor inoculation, pretreatment with low doses of cyclophosphamide, and therapy using the attenuated strain of Bacillus Calmette-Guerin (BCG). In addition to providing potential approaches toward effective inhibition of tumor growth, experiments utilizing such agents have allowed greater insight into the complex host regulatory mechanisms which maintain a balanced immune response.

Materials and Methods

Animals

A/J (H-2^a) mice were obtained from Jackson Laboratories (Bar Harbor, Me).

Tumors

The S1509a and SAI tumors were used. Their culture and maintenance requirements have been previously described.[2]

Antiserums

Batch 482 of BIO.A(3R) anti-BIO.A(5R) and Batch 480 of (DBA/2 × BIO.A[3R])F_1 anti-BIO.A(5R), hereafter designated anti-I-J^k, were kindly provided by Dr. M. E. Dorf (Harvard Medical School) and have been described elsewhere.[5,6] The preparation of rabbit antimouse thymocyte serums (ATS) has also been previously described in detail.[2]

Cyclophosphamide

Normal A/J mice received an intraperitoneal injection of 5 mg/kg cyclophosphamide (Cytoxan, Mead Johnson, Evansville, Ind) diluted in normal saline 3 days prior to tumor inoculation.

BCG

The attenuated strain of *Mycobacterium bovis*, in the form Bacillus Calmette-Guerin, was obtained from the Pasteur Institute (Paris). Colony counts were kindly performed by

Dr. K. D. Stottmeier (Mattapan Chronic Disease Hospital, Massachusetts) and found to equal 1.6×10^6 viable bacteria per milliliter.

Tumor Inoculation and Measurement

Appropriate numbers of washed, cultured tumor cells were suspended in 0.1 ml Hanks' balanced salt solution and inoculated subcutaneously in the shaved backs of A/J mice. In experiments utilizing BCG, tumor cells were suspended directly in 0.15 ml of BCG suspension in saline and similarly inoculated. Tumors were examined on the days indicated, and tumor size was determined by measurement in two directions using vernier calipers.

Statistical Significance

The statistical significance of differences in mean tumor size between groups consisting of 5 mice was calculated according to the Student *t* test. Standard errors of the means are also indicated.

Results

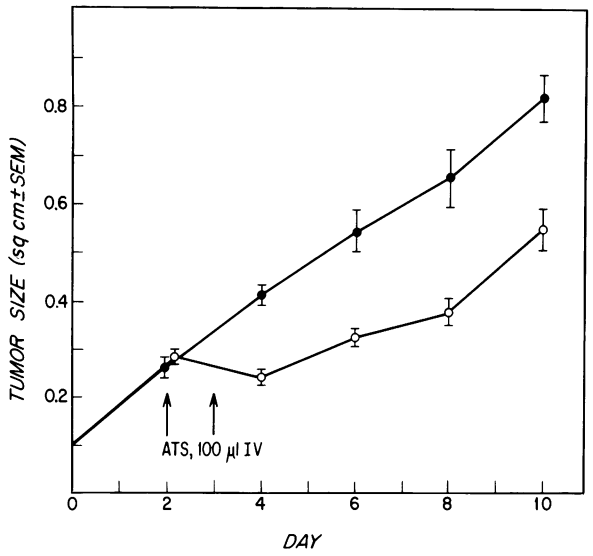
The Effect of Antithymocyte Serums (ATS) on Tumor Growth

It has previously been reported that administration of 200 μl of antithymocyte serum (ATS) on the day of inoculation with 10^6 S1509a cells (Day 0) and on Day 1 results in slightly enhanced tumor growth in normal animals compared with untreated controls.[2] In contrast, administration of the same volume of ATS on days 3, 4, 6, 8, and 10 following tumor cell inoculation caused an inhibition of tumor growth. The discrepancy of the effect of ATS is thus dependent on the protocol of treatment and probably reflects the ATS-sensitive T-cell population predominating at the time of and subsequent to tumor challenge.

The precise regimen of antiserum treatment required to achieve reduced tumor growth has been further defined in the present study. One hundred microliters of ATS was given to normal A/J mice on Days 2 and 3 following a 10^6 S1509a challenge. The effect of this treatment on tumor development is depicted in Text-figure 1. As in the previous study, this protocol for postinoculation administration of antiserums also resulted in an inhibition of tumor growth in treated compared with control animals. These results suggest that the major effect of ATS administered after tumor initiation is the elimination of a population of suppressor cells, with little interference with effector cell activity.

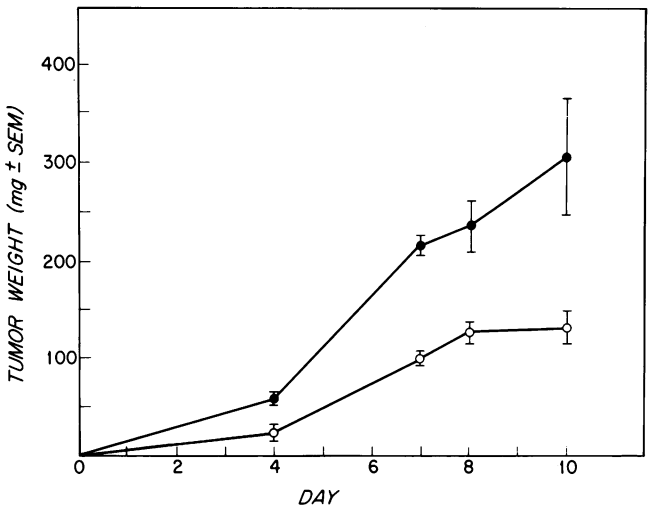
The Effect of Anti-I-J Therapy on Tumor Growth and Incidence

A more selective approach to the elimination of suppressor cells in the tumor-bearing host has been described previously.[5] Administration of small quantities of antibodies directed to interact with I-J determinants on



TEXT-FIGURE 1—The effect of antithymocyte serums (ATS) on S1509a tumor growth in nonimmune A/J mice. A/J mice were given 10^6 S1509a cells subcutaneously on Day 0. One group of mice received intravenously 100 µl ATS/day/mouse for 2 consecutive days beginning on the second day after tumor implantation (*open circles*). Another group received Hanks' solution (*solid circles*). Normal rabbit serums which had been absorbed with S1509a cells had no demonstrable effect (data not shown). Differences between groups are highly significant ($P < 0.005$) on Days 4, 6, 8, and 10.

suppressor cells and suppressor factors leads to significantly diminished tumor growth in primary tumor-bearing animals.[5,6] As can be seen in Text-figure 2, 2 µl of anti-I-J^k treatment per day leads to markedly limited tumor development as deduced from the weights of resected tumors in anti-I-J–treated or control groups of tumor-bearing mice. These results are in precise accord with our previously published results on tumor size.[1-6] We now extend these results to demonstrate the effect of anti-I-J treatment on the initial appearance of tumors when suboptimal doses of

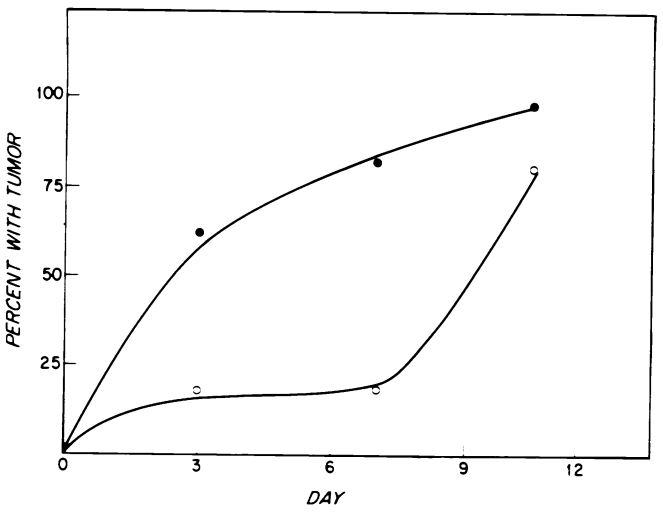


TEXT-FIGURE 2—The effect of anti-I-J alloantiserums on S1509a tumor growth in nonimmune A/J mice. A/J mice were given 10^6 S1509a cells subcutaneously. One group of mice received 2 µl of a (DBA/2 × 3R)F_1 anti-5R [anti-I-J^k] antiserum/day/mouse (*open circles*). Another group of mice received Hanks' solution (*solid circles*). At the days designated, tumors were excised, trimmed, and weighed. Statistical comparison of the groups was significant on Days 4, 7, 8, and 10.

tumor cells are used. Inoculation with 10^5 to 10^6 S1509 or SAI cells consistently results in 100% tumor incidence in normal syngeneic recipients, whereas a challenge of 10^4 tumor cells typically results in tumor induction in only 80% of normal animals. The effect of administration of 2 μl anti-I-J^k/day on the development of tumors resulting from 10^4 S1509a challenge is depicted in Text-figure 3. Animals treated with anti-I-J antiserums exhibited a delayed rate of tumor appearance compared with untreated controls. That is, only 20% (1/5) of anti-I-J^k-treated animals displayed palpable tumors by 8 days after inoculation, while 80% (11/15) of control animals were tumor-positive by this time. At a later time (Day 12) this difference was no longer apparent. Thus, administration of antiserums designed specifically to interfere with suppressor cells and suppressor factors limits not only the rate of growth but also the rate of appearance of tumors when threshold doses of tumor cells are used.

Reduction of Tumor-Specific Suppressor Activity by Cyclophosphamide

In an attempt to further analyze the role of suppressor cells in the regulation of tumor development, we examined the efficiency of cyclophosphamide (CY) treatment as a means of reducing suppressive activity in tumor-bearing animals. It has already been established that administration of low doses of CY (5 to 20 mg/ml) results in enhanced antibody [7,8] and delayed hypersensitivity [9] responsiveness, presumably due to the elimination of suppressor cell precursors, while higher doses (>200 mg/kg) of CY may depress antibody formation through a direct effect on B



TEXT-FIGURE 3—The effect of anti-I-J antiserums on tumor appearance and incidence. Anti-I-J^k antiserums (3R anti-5R, 2 μl/day/mouse) were administered each day to A/J mice beginning at the time of a 10^4 S1509a cell inoculum (*open circles*). Another group of A/J received Hanks' solution in lieu of antiserum (*solid circles*).

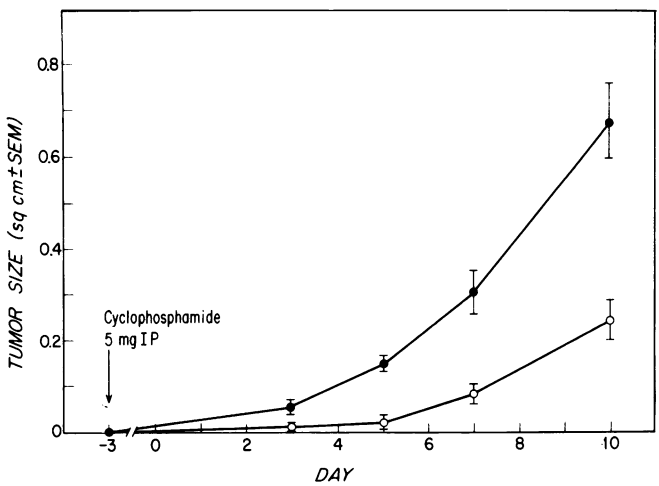
cells.[10] Based on these observations, the effect of low-dose CY pretreatment on tumor development in a system known to be under suppressor cell regulation was investigated. Results of one such experiment are shown in Text-figure 4.

Pretreatment of normal animals with 5 mg/kg CY 3 days prior to inoculation with 10^5 SAI resulted in a delay in tumor appearance and an inhibition of tumor growth compared with untreated controls. These results are compatible with a decrease in suppressor cell activity by elimination of suppressor precursors, leaving a population consisting primarily of pre-helper or pre-killer cells. This latter assumption was further tested by adoptive transfer of spleen cells from CY-pretreated normal animals into normal recipients at the time of tumor inoculation.

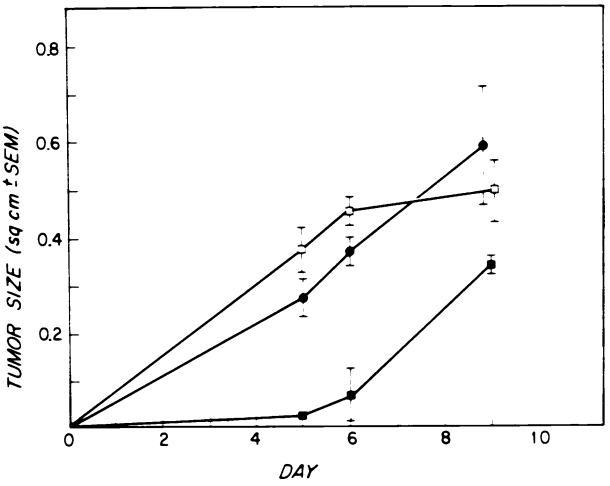
As seen in Text-figure 5, transfer of 5×10^7 normal spleen cells, including potential suppressor as well as helper-type cells, has no significant effect on tumor growth in normal animals receiving a 10^5 SAI inoculum. Transfer of equal numbers of splenocytes from CY-pretreated normal donors, however, led to delayed appearance and decreased tumor growth in normal recipients. These results support the notion that low-dose CY pretreatment eliminates a population of cells capable of induction into suppressor pathways, allowing greater expression of host effector responses.

Augmentation of Cytolytic Activity in Tumor-Bearing Hosts

Another approach to the inhibition of *in vivo* tumor development lies in the augmentation of effector mechanisms as opposed to the reduction of

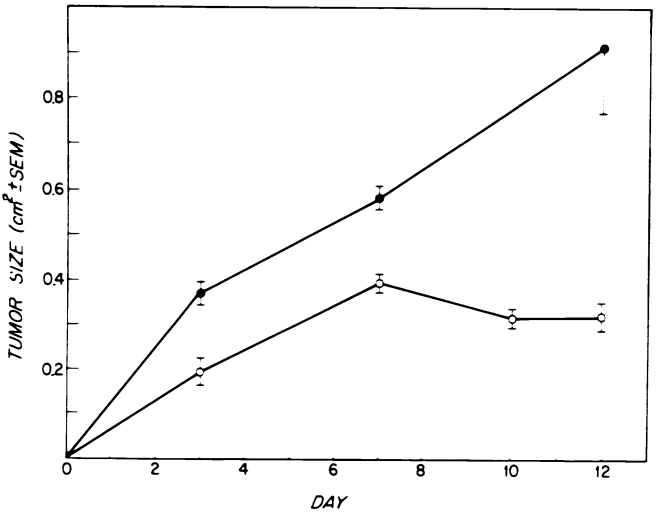


TEXT-FIGURE 4—The effect of low-dose cyclophosphamide on tumor growth. Normal A/J mice were given 10^5 SAI cells subcutaneously on Day 0. One group of mice had received cyclophosphamide at a dose of 5 mg/kg intraperitoneally 3 days previously (*open circles*). The control group received only 10^5 SAI cells on Day 0 (*solid circles*).



TEXT-FIGURE 5—The effect of transfer of splenocytes obtained from animals treated with low-dose cyclophosphamide. Non-immune A J mice received 10^6 SAI cells subcutaneously on Day 0. One group received 5 × 10^7 normal A J splenocytes mouse intravenously (*open squares*); another group received 5 × 10^7 splenocytes intravenously, which were obtained from A J mice treated with 5 mg kg of cyclophosphamide 3 days previously (*solid squares*). A third group received Hanks' solution in lieu of cells (*circles*).

suppressor activity. One such mode of therapy which we have investigated involves the administration of the attenuated form of the tubercle bacillus Bacillus Calmette-Guerin (BCG). We studied the ability of BCG to interfere with tumor growth in the S1509a system. Preliminary experiments revealed that the most efficient method of applying BCG therapy consisted of suspending the tumor cells destined for subcutaneous inoculation directly in a solution of live BCG organisms. Results of a representative experiment utilizing this protocol and a 10^6 S1509a inoculum are depicted in Text-figure 6.



TEXT-FIGURE 6—The effect of intralesional BCG on S1509a tumor growth. A group of 5 normal mice was given 10^6 S1509a cells subcutaneously (*solid circles*). Another group of 5 mice received the same number of S1509a cells suspended in BCG (*open circles*). Comparison of tumor size was statistically significant on all days.

Animals receiving BCG plus tumor cells exhibited a decreased rate of tumor development compared with control animals. Tumors in the treated group reached peak size at Day 6 and remained stable thereafter. Untreated control animals exhibited 100% mortality as a result of progressive tumor growth by 35 ± 10 days after inoculation; mice given BCG plus tumor remained alive more than 90 days after treatment, with only a small tumor nodule still visible. In data not shown, we studied the effect of daily therapy with anti-I-J^k antiserum in combination with BCG treatment. Although anti-I-J treatment resulted in a transient reduction in the rate of tumor growth above that observed with BCG alone, this synergy was no longer apparent by 14 days after inoculation, and animals from both groups exhibited similar rates of survival. Thus, BCG used under defined conditions can depress tumor development to an extent compatible with long-term survival in a murine system.

Discussion

Regulation of host immune responsiveness to antigens expressed on certain chemically induced tumors has been shown to involve antigen-specific suppressor T cells.[1-6] Such suppressor cells inhibit the efficiency of effector responses generated against the syngeneic tumor, as evidenced by the ability of suppressor cells or suppressor-cell–derived suppressor factors to limit an ongoing immune response.[3,4] Therefore, the diminution or elimination of suppressor cell activity *in vivo* constitutes a rational approach to the immunotherapeutic treatment of malignancies suspected of being under suppressor cell regulation.

Characterization of suppressor cells controlling antibody responsiveness has shown them to bear determinants encoded by the I-J subregion of the murine MHC.[11] Such determinants have also been found on tumor-specific suppressor factors.[12] Thus, it has been demonstrated that *in vivo* administration of antiserums directed against I-J region coded products leads to a reduction in the growth of certain methylcholanthrene-induced syngeneic tumors.[5,6] We have extended these results to show that anti-I-J treatment leads to reduced tumor mass, as determined by weight. These results corroborate precisely with the previously described experiments on tumor size.[5] We have also shown that such treatment results in a delayed rate of tumor appearance when suboptimal doses of tumor are given. Animals treated with anti-I-J antibodies are no longer capable of adoptively transferring suppression to immune recipients [5] and on histologic analysis demonstrate a degree of leukocytic infiltration into the tumor, more closely approximating an immune than a primary response.[6] Attempts to interfere with S1509a or SAI tumor growth using antiserums

with known anti-gp 71 activity [13] have been unsuccessful.[14] Hence, this observation, coupled with the loss of suppressor activity after I-J treatment, intimates that the mode of action of anti-I-J serum relates to its interaction with suppressor cells or their products and is not a result of contaminating antiviral activity in this serum. It can be inferred from the observed ability of anti-I-J treatment to effect a delay in the appearance of primary tumors that suppressor cells may be involved in limiting the generation of host effector activity as well as the inhibition of the effector reactivity in an ongoing immune response.[1-4]

It is also possible to diminish suppressive activity by administration of antiserum directed against more generally expressed lymphocyte markers, in this case an xenoantiserum directed against antigens on murine thymocytes. Treatment of tumor-bearing animals with ATS for only short periods during the early phase of tumor development is sufficient to reduce the rate of tumor growth for several days subsequent to therapy. In contrast is the effect of ATS administered simultaneously with and 1 day following tumor challenge.[1,2] Tumor growth in animals treated according to this schedule is slightly enhanced rather than inhibited. This differential response to ATS may relate to the nature of the antibody-sensitive population predominating at the time of treatment. Cells differentiating along effector pathways may be preferentially inhibited by antibodies administered at the time of tumor challenge, whereas suppressor type cells, which have been detected by 24 hours after inoculation,[3,4] are the major target of antithymocyte antibodies injected 2 or 3 days following tumor inoculation. Therefore, the timing of antiserum administration relative to the stage of tumor development is critical to achieve a growth inhibitory rather than stimulatory effect. The overall action of ATS when given at later times during tumor progression has not been investigated in this system.

Interference with the development of suppressor cell precursors provides an additional means of defining more precisely the suppressor cell circuit [15,16] regulating syngeneic tumor development. Results in this and several nontumor systems [7,8,17] indicate that this can be accomplished by pretreatment *in vivo* with low doses of cyclophosphamide. Thus, administration of CY 3 days prior to tumor challenge leads to a significant reduction in the rate of tumor growth in treated animals. This effect has been corroborated by recent data demonstrating a delay in the appearance of tumors induced by methylcholanthrene in CY-treated mice.[18] CY pretreatment of normal animals prior to an adoptive transfer results in augmented effector activity in primary tumor-bearing recipients, which is manifest as decreased tumor growth. Thus, the elimination of a popu-

lation of cells with the potential to express suppressor activity leaves a population capable of differentiating primarily along helper and effector pathways. This knowledge is of potential value in clarifying the sequence of events involved in the expression of suppression, as well as its obvious therapeutic relevance. Further examination of this system is required to determine the relationship between the CY and anti-I-J sensitive suppressor populations.

Augmentation of host effector activity, with or without simultaneous inhibition of suppressive activity, constitutes another approach to the *in vivo* reduction of tumor development. BCG-based immunotherapy has proved successful in potentiating T-dependent responses to the P815 mastocytoma [19] and can induce nonspecific macrophage antitumor cytotoxicity.[20] As reported here, BCG treatment can lead to markedly reduced tumor growth *in vivo* with significant prolongation of host survival.

Thus, various techniques have been described which can be applied to the *in vivo* inhibition of suppressive influences and stimulation of effector mechanisms. It should be possible, by coordinating specific combinations of therapy, to shift the balance of host reactivities toward pathways most beneficial for the elimination of a growing neoplasm.

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